

AXIMA™

SNP analysis performed on a research grade MALDI-TOF mass spectrometer

- Utilizes the simplest SNP analysis format with minimized reagent costs
- Multiplex reactions can be analyzed simultaneously
- Extending the utility of the research grade instrument

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Introduction

The Human Genome Project was actively conducted on an international scale over recent years to decode the base sequence in the “genome” that contains the entire genetic information of *homo sapiens*. It was reported in 2000 that the project had succeeded in identifying almost all the 3 billion base pairs (sequence of the bases A, G, C, T) in the genome. Along with the analysis of the detailed genome sequence, attention was focused on the analysis of SNPs (Single Nucleotide Polymorphisms). A single nucleotide polymorphism results when a single base in the sequence is replaced by another base. It is estimated that the genome of any individual includes 300,000 to 10 million SNPs.

In a living organism, three letters representing bases in the genetic code provide the information for one amino acid. Proteins with various functions are formed by specific amino acids in a certain order. As the base sequence determines the order of the amino acids forming a protein, the replacement of a single base may result in a protein that does not function correctly. This can produce differences in the individual’s constitution: e.g. ability to metabolize alcohol; drug effectiveness for the treatment of a condition; or disease susceptibility. It is anticipated that examination of an individual’s SNP profile could provide valuable information in understanding these features. Such developments give promise to 21st Century goals for tailor-made treatments and personalized medicine.

As the positions of SNPs resulting in likelihood of hypertension or cancer, for example, are already known, a method is needed to rapidly evaluate which base is replaced at specific SNPs in an individual. Here we present examples of the genotyping (base identification) of known SNPs using the AXIMA-CFR™ MALDI Mass Spectrometer.

Principle

Figure 1 shows sample preparation for SNP genotyping. This is a method known as “single nucleotide primer extension” (SNUPE), introduced by Kuppuswamy et al in 1991¹.

- (1) Amplify the DNA fragment containing the SNP using PCR.
- (2) Create a primer adjacent to the SNP in the PCR-amplification product obtained at step (1).
- (3) Conduct SNUPE.

Mix the enzyme ThermoSequenase™ (Amersham Biosciences) DNA Polymerase (0.3 unit/μL), template from step (1) (12.5 pmol/μL), SNUPE primer (1 pmol/μL) and the appropriate two ddNTPs and react in a thermal cycler.

Following incorporation of one of the ddNTPs the primer cannot extend any further.

(ddNTP = di-deoxynucleotidetriphosphate)

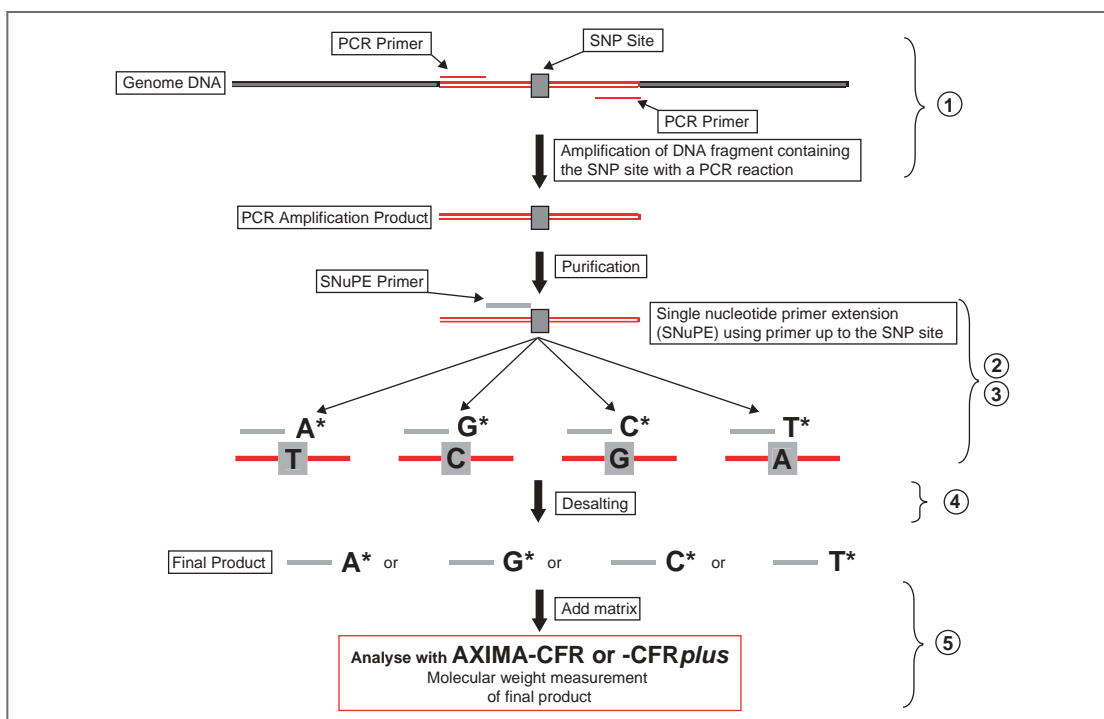


Figure 1. Single Nucleotide Primer Extension (SNUPE) protocol

PCR conditions: 95°C, 30 seconds -> 50°C, 30 seconds -> 70°C, 4 minutes (25 cycles)

- Purify the primer and the SNuPE reaction product(s).

For desalting, use ZipTip® C18 (manufactured by Millipore), column purification, or ethanol precipitation without sodium salts.

- Mix and analyze the sample and matrix on the AXIMA-CFR™ sample plate.

Example 1 - homozygotes

ras Mutant Set c-Ha-ras codon 12 (manufactured by Takara Shuzo Co., Ltd.) was used as the test model.

A matrix of 0.7 M 3-hydroxypicolinic acid (3-HPA) and 0.07 M ammonium citrate saturated solution in 50% acetonitrile was desalted prior to analysis. Analysis involved negative ion detection in the AXIMA-CFR™ reflectron mode. Figure 2 shows examples of analysis of each sample. The SNuPE primer was an 18 mer. A mass spectrum was obtained for the final reaction product for each sample, which was extended by just one base from the primer. Table 1 shows the theoretical molecular weight of each ddNTP. The base incorporated onto the SNP primer was readily confirmed from the difference in molecular weight between the primer and SNuPE reaction product in Figure 2.

Example 2 - heterozygotes

The template gene containing a particular SNP is often heterozygote. In this case, a SNP site might be a different nucleotide base and following analysis the resulting extension products would have different masses. Such a result is shown in Figure 3. The samples and matrix used were as described for Example 1. After desalting, TOF-MS analysis was conducted in the AXIMA-CFR™ using reflectron mode to detect negative ions.

The SNuPE primer used for this analysis was a 24 mer (MW: 7231.45). As the sample was heterozygous, multiple final products were obtained. The base sequence at the SNP site could be identified from the difference in molecular weight between the primer and two SNuPE reaction products. In addition, extension with ddT or ddA with a mass difference of only 9 Daltons (see Table 1) could be clearly determined. Consequently, genotyping of heterozygous samples was possible.

Example 3 - multiplexed SNuPE primers

Simultaneous genotyping of multiple SNPs is also possible, since more than one copy of a given SNP heterozygosity e.g. A/T may be present on one gene or PCR product SNP template. In this case, multiplex primers with different lengths can be prepared to

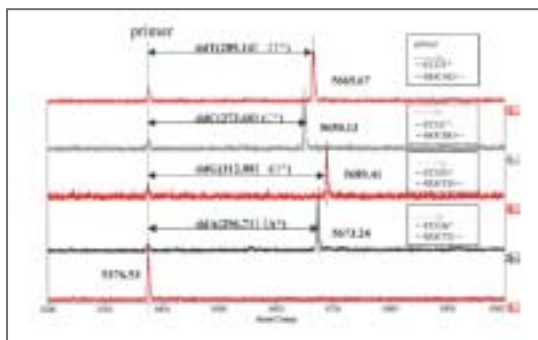


Figure 2. SNuPE analysis of homozygotes

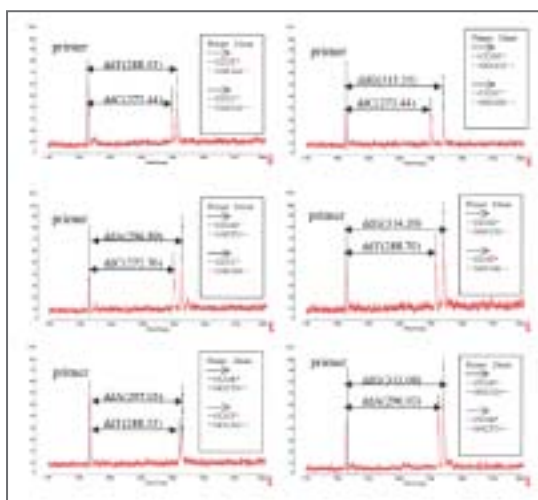


Figure 3. SNuPE analysis of heterozygotes

	Theoretical Molecular Weight
dd A	297.2
dd G	313.2
dd C	273.2
dd T	288.2

Table 1 Theoretical molecular weight of ddNTP

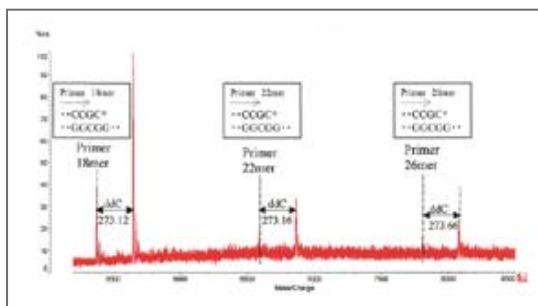


Figure 4. SNuPE analysis of homozygotes using a multiplex primer

allow simultaneous SNuPE, analysis, and genotyping. Figure 4 shows an example of this. SNuPE primers of three different sizes (18 mer, 22 mer, and 26 mer) were mixed together to conduct simultaneous SNuPE. A single mass spectrum was obtained for the final reaction product for each primer, which was extended by just one base from the primer. The base sequence at each SNP site was readily confirmed from the difference in molecular weight between the primer and SNuPE final product.

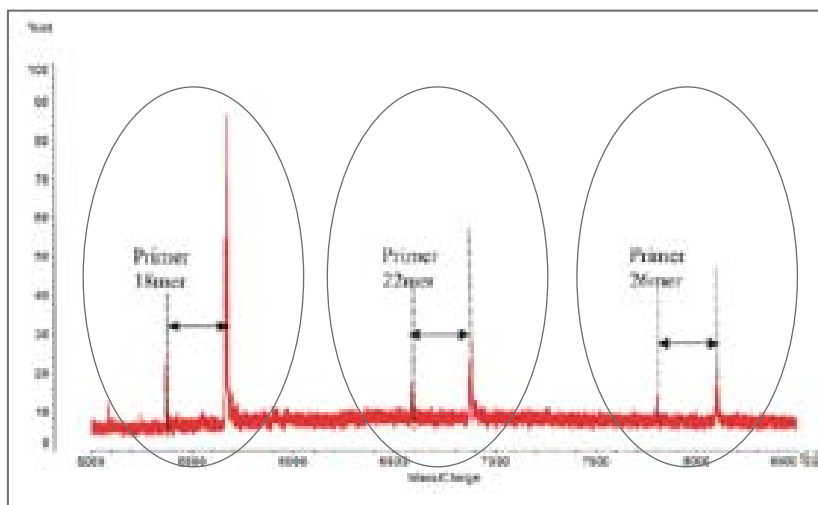
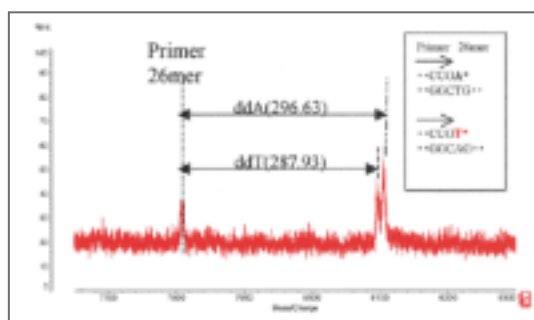
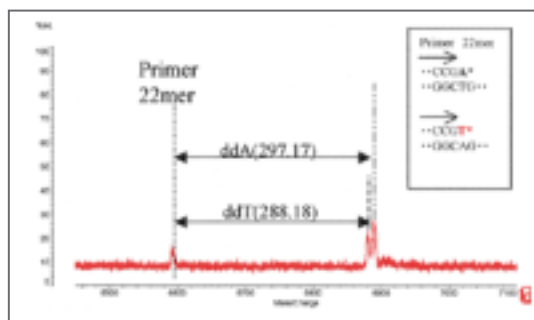
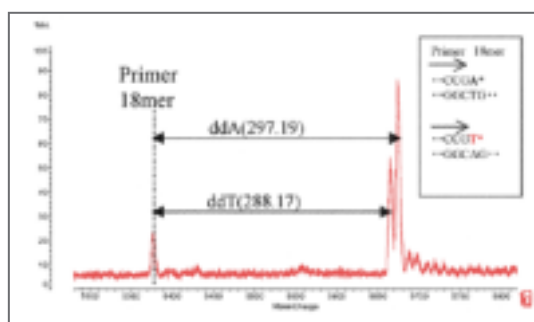


Figure 5. SNP analysis of heterozygotes using a multiplex primer

Figure 5 shows an example of a heterozygous sample. A mass spectrum was obtained from the primer for each reaction product. Zoom views of the target mass spectrum show that multiple signals could be obtained from this single mass spectrum. From the difference in molecular weight between the primer and SNuPE final product, multiple genotyping of heterozygotes could be conducted simultaneously at multiple positions.



Conclusion

- using the AXIMA-CFR™ it was possible to determine homo- and hetero-zygotic variants of SNPs. The 9 Da mass difference between A and T termini was distinguished
- SNP reactions could be "multiplexed" so that the results from combined SNuPE reactions with the same possible heterozygosity could be determined directly in one mass spectrum
- the microtitre plate design of the AXIMA™ sample target allows a direct relationship between the usual 96 / 384 reaction PCRs performed in molecular biology labs and the final mass analysis step
- the user has the option of either positive or negative ionization allowing flexibility in choice of matrix and a dedicated SNP software module enabling sample identification input, result reporting and analysis output to external database tools

BIBLIOGRAPHY

1. Kuppaswamy, M.N. et al., Proc. Natl. Acad. Sci. USA 88, (1991), 1143-1147

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